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Differential detection of *Hammondia hammondi* from *Toxoplasma gondii* using polymerase chain reaction

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Abstract

Hammondia hammondi and *Toxoplasma gondii* are two related coccidian parasites, with cats as definitive hosts and warm-blooded animals as intermediate hosts. It is difficult to differentiate them by morphological and serological parameters. In the present study, primers were designed to specifically amplify the ITS-1 region of *H. hammondi* to differentiate it from *T. gondii*. Attempts were made to detect the presence of *H. hammondi* DNA in the tissues of mice infected with *H. hammondi* alone, as well as from mixed infections with *T. gondii*, using the newly designed primers. The de novo primers effectively amplified the *H. hammondi*-specific target fragment from all samples containing *H. hammondi*, including those with concomitant *T. gondii* infection. Further, the primers did not amplify any fragment from the related parasites like *T. gondii*, *Neospora caninum* and *Hammondia heydorni*. The new primers provide simple and efficient means to differentially diagnose *H. hammondi* from *T. gondii* even in samples containing both parasites, thus obviating the need for other labourious techniques like mouse bioassay and in vitro cultivation.
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Keywords: *Hammondia hammondi*; *Toxoplasma gondii*; ITS-1; PCR; Diagnosis

1. Introduction

Hammondia hammondi is a coccidian parasite of cats with an obligatory heteroxenous life cycle involving rodents and other warm-blooded animals [1]. It is closely related to *Toxoplasma gondii.* Although *H. hammondi* and *T. gondii* share many hosts [2,3], *H. hammondi* has not been associated with any disease manifestation in any of these hosts. There are no major morphological differences between any of the stages of *H. hammondi* and *T. gondii* [4,5]. These two related parasites also elicit antibodies recognized by most serological tests [6] including modified agglutination test (MAT). This morphological and phylogenetic similarity has until now created uncertainty and debate about the taxonomic position of *H. hammondi* [7,8]. However, the parasite has epidemiological significance due to its close resemblance to *T. gondii*, owing to the fact that *T. gondii* is pathogenic to man and animals, and cat is the definitive host for both organisms. Thus, it is imperative that these infections are differentially diagnosed. Mouse bioassay, and to a lesser extent, in vitro growth characteristics, are the two techniques available to differentiate these two organisms. Unlike *T. gondii*, which can be continuously passaged in mice, only the oocysts of *H. hammondi* are infective to mice and it cannot be maintained beyond the first mouse passage. Similarly, all stages of *T. gondii* can infect cells in vitro and the parasite can be maintained as tachyzoites indefinitely in culture. In contrast, only sporozoites and tachyzoites of *H. hammondi* are infective to cultured cells in which they form tissue cysts and cannot be passaged continuously [4,9]. However, these techniques are time and labour intensive. Further, neither of these

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techniques can diagnose mixed infection, as *T. gondii* will supervene mouse or in vitro passage, effectively masking the presence of *H. hammondi* infection.

Here, we describe a PCR assay, based on amplification of the ITS-1 region, for the specific detection of *H. hammondi* in samples with mixed infection containing *T. gondii*.

2. Materials and methods

Isolates of *H. hammondi* (HH 34, [4]), *Hammondia heydorni* (ARG 51, [10]) and *T. gondii* (VEG strain, [11]) were available as sporulated oocysts. *Neospora caninum* (NC1, [12]) was maintained in vitro in CV1 cells. The oocysts ($\sim 10^6$) were treated with 0.525% bleach and DNA was extracted using DNAzol [13], while DNA was extracted from tachyzoites using DNAeasy tissue kit (Qiagen, Valencia, CA) as per manufacturer's instruction. The stock DNA was prepared in a volume of 50 μl of elution buffer. This was diluted 10-fold and 2 μl was used for PCR.

A group of 5 out bred Swiss Webster mice was infected with 10^4 oocysts *T. gondii*. A group of 5 interferon-gamma gene knock out (KO) mice [4] was infected with 10^4 oocysts of *H. hammondi*, while a mixture of 10^4 *H. hammondi* and 10^3 *T. gondii* oocysts were administered to another group of 5 KO mice. Mesenteric lymph nodes were collected from the mice that died due to acute infection and the impressions (both unstained and those stained with Giemsa) were examined for the presence of tachyzoites. The KO mice were killed 6 weeks after infection, and a small piece of the quadriceps muscle was collected and compressed under the coverslip. These squash preparations were examined under 10× objective of a microscope for the presence of tissue cysts [4]. Tissue extracts from all mice in a group were pooled and DNA extracted using DNAeasy tissue kit in a volume of 50 μl. For the validation of PCR assays, 2 μl of a 10-fold dilution of the stock DNA was used.

Primers were designed to specifically amplify a portion of the ITS-1 region of *H. hammondi*. The ITS-1 sequences of *H. hammondi* (**AF076857**), *H. heydorni* (**AY579761**), *N. caninum* (**AY582109**) and *T. gondii* (**AY582110**) were aligned using GENETOOL software. The forward primer was the same (CT1, [13]) that was used for the amplification of ITS-1 fragments from all toxoplasmatiid DNA. The reverse primer (HhammITS) specific to *H. hammondi* was designed to incorporate at its 3′ end (Fig. 1), the 2 polymorphic nucleotides (AG) of *H. hammondi*, which differentiates it from *T. gondii* (GA). Similarly, a reverse primer (ToxoITS) was designed to specifically amplify *T. gondii,* by incorporating to its 3′ end, the 2 polymorphic nucleotides (AA) of *T. gondii*, which differ at those positions from that of *H. hammondi* (GG). The reverse primer CT2 [13] was used with CT1 to amplify the ITS-1region from all toxoplasmatiid (*T. gondii*, *H. hammondi*, *H. heydorni* and *N. caninum*) DNA.

PCR assays were standardized to amplify the target fragment of 339 bp from *H. hammondi* oocyst DNA (using the primer set CT1/HhammITS) and the 294 bp fragment from *T. gondii* oocyst DNA (using the primer set CT1/ToxoITS). The DNA from other toxoplasmatiids, including *N. caninum* and *H. heydorni*, as well as water, was used as negative controls. The quality of the negative controls (except water) was ascertained by the amplification of the respective ITS-1 fragments using the common toxoplasmatiid primers CT1/CT2 [13]. The PCR assays (25 μl) were setup using 1 U *Taq* (Invitrogen, Carlsbad, California) 200 μM each of the dNTPs and 15 pmol of each primer. As a routine, 2 μl of the DNA working solution was added to each tube. The thermal cycling protocol followed included an initial denaturation of 95° C, for 5 min followed by 10 cycles of 95° C for 30 s, 65° C for 30 s, 72° C for 1 min, 10 cycles of 94° C for 30 s, 62° C for 30 s, 72° C for 30 s and 15 cycles of 94° C for 30 s, 60° C for 30 s, 72° C for 30 s, followed by a final extension of 72° C for 5 min.

For validating the do novo PCR assays, DNA samples extracted from the mice tissues, along with the respective positive (*T. gondii* and *H. hammondi*) and negative (*H. heydorni*, *N. caninum* and water) controls were tested for amplification using toxoplasmatiid, *H. hammondi*-specific and *T. gondii*-specific PCR assays. The amplification products were resolved in a 2% agarose gel and documented.

3. Results and discussion

Tachyzoites could be demonstrated microscopically in the mesenteric lymph node extracts from mice that died due to acute disease. Tissue cysts of *H. hammondi* could be demonstrated in the muscle squash preparations from mice killed 6 weeks after infection with oocysts of *H. hammondi* alone.

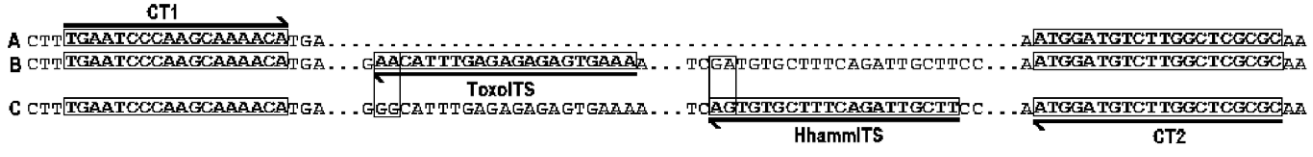

Fig. 1. Design of primers (bold half arrows) for the amplification of ITS-1 region from toxoplasmatiid (A), *Toxoplasma gondii* (B) and *Hammondia hammondi* (C) DNA. The primer pair CT1/CT2 amplifies the ITS-1 region from all toxoplasmatiids, including *T. gondii*, *H. hammondi*, *Hammondia heydorni* and *Neospora caninum*. The primer pair CT1/ToxoITS is specific for *T. gondii*, while CT1/HhammITS amplifies only *H. hammondi* ITS-1. Note that the specific primers are designed to include the 2 polymorphic nucleotides (boxed area) at their 3′ end.

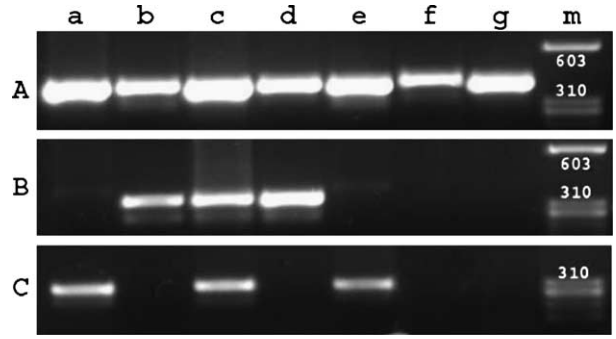

Fig. 2. Amplification of ITS-1 region using toxoplasmatiid (A), *H. hammondi*-specific (B) and *T. gondii*-specific (C) primers. DNA from *T. gondii* (a) and *H. hammondi* (b) and tissues of mice infected with both *T. gondii* and *H. hammondi* (c), *H. hammondi* alone (d) and *T. gondii* alone (e), along with negative controls *N. caninum* (f) and *H. heydorni* (g) were used. The common toxoplasmatiid primers amplified the approximately 400 bp fragment from all samples (A), indicating the presence of amplifiable DNA. The *H. hammondi*-specific primer pair amplified the target amplicon of 339 bp from positive control (Bb), single infection of *H. hammondi* (Bd) and mixed infections containing both DNA (Bc), but not from *T. gondii* (Ba, Be), *H. heydorni* (Bf) or *N. caninum* (Bg). The *T. gondii*-specific primer pair does not cross amplify any of the *H. hammondi* DNA (Cb, Cd). m—Molecular weight marker $\varnothing \times \lambda$ DNA-*Hae* III digest.

The results of amplification using the different primer sets are depicted in Fig. 2. Amplification of the approximately 400 bp PCR product was achieved from *T. gondii*, *H. hammondi*, *N. caninum* and *H. heydorni* DNA when the common toxoplasmatiid primers (CT1 and CT2) were used (Fig. 2A). Amplification of the *H. hammondi*-specific 339 bp product was achieved only with the DNA from *H. hammondi* (Fig. 2B), while only samples containing *T. gondii* DNA showed amplification of its specific target fragment of 294 bp (Fig. 2C).

Successful amplification of the common toxoplasmatiid fragments from *T. gondii*, *H. hammondi*, *H. heydorni* and *N. caninum* DNA indicated the presence of adequate quantity of amplifiable DNA in these samples. The common toxoplasmatiid primers can thus be very useful in detecting the presence of any of the toxoplasmatiid organisms, except *Besnoitia* sp.

Only those samples, which contained *H. hammondi* DNA, resulted in successful amplification of the *H. hammondi*-specific fragment. No products were amplified from the DNA of related organisms, particularly from those of *T. gondii* and *H. heydorni*. Thus, the primer pair was found to be specific to *H. hammondi*. Additionally, from samples that contained both *H. hammondi* and *T. gondii* DNA (Fig. 2, lane c), both the *H. hammondi* and *T. gondii*-specific fragments could be separately amplified using the specific sets of primers, CT1/HammITS and CT1/ToxoITS, respectively.

There is overwhelming biological evidence now [4] pointing to the validity of *H. hammondi* as a separate entity from *T. gondii*. The design of specific primers to diagnose *H. hammondi* further augments the validity of the species and facilitates investigations into the biology of the organism.

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